# An approach for establishing Oncomine Focus Assay performance

## Introduction

Cancer is understood to be a disease of the genome with different hallmarks that include genome instability and an accumulation of somatic mutations. While cancers are characterized by numerous genomic aberrations, some of these somatic mutations, known as driver mutations, induce growth and impaired differentiation leading to cancer development. The identification of these driver mutations in a tumor enables the development of targeted therapies directed against the specific molecular alterations driving the tumor.

Recent advances in genome sciences, including next-generation sequencing (NGS), have led to the identification of hundreds of somatically altered genes through the analysis of tens of thousands of cancer samples from individual investigators and large consortia, such as The Cancer Genome Atlas (TCGA). These technological advances are also changing routine molecular pathology research from single gene–based tests (e.g., Sanger sequencing to assess *EGFR* mutations in lung tumor samples) to multiplexed NGS assays.

Next-generation sequencing solutions that can assess all classes of relevant targets, including point mutations, short insertions or deletions (indels), copy number variants (CNVs), and gene fusions from formalin-fixed, paraffin-embedded (FFPE) tissues or fine-needle aspirates may help advance cancer research toward a future in which precision medicine approaches for all cancer types are rapid, inexpensive, and scalable. To enable this vision, technical solutions should be coupled with dynamic, scalable, and analytical approaches capable of prioritizing future treatment options.

The Ion Torrent™ Oncomine™ Focus Assay* is a multibiomarker NGS assay that enables the detection of variants in 52 key solid tumor genes. These genes are well characterized in the published literature and associated with oncology drugs that are FDA approved, part of National Comprehensive Cancer Network (NCCN) guidelines, or in clinical trials. The assay allows concurrent analysis of DNA and RNA to simultaneously detect multiple types of variants, including hotspots, single nucleotide variants (SNVs), indels, CNVs, and gene fusions, in a single workflow. The Oncomine Focus Assay is based on Ion AmpliSeq™ technology and is designed for use with the Ion PGM™ System, but is also compatible with other Ion Torrent™ sequencing platforms.

Here we describe a study to determine the performance of the Oncomine Focus Assay in detecting known variants present in commercially available reference and FFPE tissue materials. The study was performed in a laboratory certified by the Clinical Laboratory Improvement Amendments (CLIA) program and accredited by the College of American Pathologists (CAP). The study design was informed by the New York State NGS guidelines for somatic genetic variant detection.

ThermoFisher SCIENTIFIC

* For Research Use Only. Not for use in diagnostic procedures.

## Materials and methods

### Gene targets

The biomarkers included in the Oncomine Focus Assay were selected based on information in the Ion Torrent™ Oncomine™ Knowledgebase, one of the world's largest collections of curated oncology data [1]. The 52 genes queried by the assay fall into the categories shown in Figure 1.

### Samples

DNA samples, cell lines, and FFPE sections were used in this study.

**The following molecular standards were used to assess DNA performance:**

- Thermo Scientific™ Acrometrix™ Oncology Hotspot Control (Thermo Fisher Scientific, Cat. No. 969056) is a mixture of >500 Catalogue of Somatic Mutations in Cancer (COSMIC) mutations (SNVs, multinucleotide variants (MNVs), insertions, and deletions) across 53 genes that are confirmed by Sanger sequencing. Of these mutations, 69 annotated variants are detectable by the Oncomine Focus Assay and were included in the performance analysis.
- The Quantitative Multiplex Reference Standard (Horizon Discovery, Cat. No. HD200) is an FFPE section from which DNA was extracted using standard procedures. DNA from the Quantitative Multiplex Reference Standard bears 11 mutations (SNVs and deletions) across cancer driver genes (*BRAF, KIT, EGFR, KRAS, NRAS,* and *PIK3CA*) that are confirmed by digital PCR. Of these mutations, 8 are above the limit of detection (LOD) for the Oncomine Focus Assay as determined in this study. Allele frequency ranged from 1% to 24.5%.

**The following samples were used as processing controls:**

- For DNA processing, the negative control was Human Genomic DNA: Male (Promega, Cat. No. G1471). As a positive control, an equal mixture of DNA extracted from the following cell lines from ATCC was used: H1975 (*EGFR* mutation), H1650 (*EGFR* mutation), HCT116 (*KRAS* mutation), and A375 (*BRAF* mutation).
- For RNA processing, the negative control was Invitrogen™ Human Lung Total RNA (Thermo Fisher Scientific, Cat. No. AM7968). As a positive control, RNA was extracted from HCC78 (*SLC34A2–ROS* fusion) and H2228 (*EML4–ALK* fusion) cell lines that were obtained from ATCC.

| Hotspot genes | | Copy number variants | Fusion drivers |
|---|---|---|---|
| 35 genes | | 19 genes | 23 genes |
| DNA | | | RNA |
| *AKT1* | *JAK1* | *ALK* | *ABL1* |
| *ALK* | *JAK2* | *AR* | *ALK* |
| *AR* | *JAK3* | *BRAF* | *AKT3* |
| *BRAF* | *KIT* | *CCND1* | *AXL* |
| *CDK4* | *KRAS* | *CDK4* | *BRAF* |
| *CTNNB1* | *MAP2K1* | *CDK6* | *EGFR* |
| *DDR2* | *MAP2K2* | *EGFR* | *ERBB2* |
| *EGFR* | *MET* | *ERBB2* | *ERG* |
| *ERBB2* | *MTOR* | *FGFR1* | *ETV1* |
| *ERBB3* | *NRAS* | *FGFR2* | *ETV4* |
| *ERBB4* | *PDGFRA* | *FGFR3* | *ETV5* |
| *ESR1* | *PIK3CA* | *FGFR4* | *FGFR1* |
| *FGFR2* | *RAF1* | *KIT* | *FGFR2* |
| *FGFR3* | *RET* | *KRAS* | *FGFR3* |
| *GNA11* | *ROS1* | *MET* | *MET* |
| *GNAQ* | *SMO* | *MYC* | *NTRK1* |
| *HRAS* | | *MYCN* | *NTRK2* |
| *IDH1* | | *PDGFRA* | *NTRK3* |
| *IDH2* | | *PIK3CA* | *PDGFRA* |
| | | | *PPARG* |
| | | | *RAF1* |
| | | | *RET* |
| | | | *ROS1* |

**Figure 1. List of genes included in the Oncomine Focus Assay.**

**The following were used as test samples:**

- Three cell lines were obtained from ATCC and used to study analytical sensitivity and establish the LOD for SNVs. The cell lines were H647 (*KRAS* p.G13D), HCT116 (*PIK3CA* p.H1047R and *KRAS* p.G13D), and *SKMEL1* (BRAF p.V600E).
- One cell line, HCC4006 with an *EGFR* exon 19 deletion (*EGFR* p.L747_A750delinsP), was obtained from ATCC and used to establish the LOD for indels.
- Two cell lines were obtained from ATCC and used to study analytical sensitivity and establish the LOD for CNVs. The cell lines were H1993 (*MET* and *CDK6* amplification) and H647 (*MYC* amplification).
- A total of 56 FFPE samples were purchased that consisted of 49 solid tumor research samples from Asterand Bioscience and 7 cell lines from ATCC (Table 1). One slide bearing a 7 µm–thick section was obtained per sample. The average tumor cellularity was 65% (range 10–100%) and the average tumor area was 137 $mm^2$ (range 5–430 $mm^2$).

Table 1. FFPE test samples.

| Tumor type | Number of samples |
|---|---|
| Brain | 4 |
| Breast | 5 |
| Cervix | 1 |
| Colon | 10 |
| Esophagus | 1 |
| Lung | 13 |
| Melanoma | 2 |
| Pancreas | 4 |
| Prostate | 5 |
| Stomach | 2 |
| Uterus | 2 |
| Cell lines (H1993, H2228, H647, HCC4006, HCT116, RKO, SKMEL-1) | 7 |
| **Total** | **56** |

### Sample processing

Nucleic acid isolation from cell lines and FFPE samples was performed with the Invitrogen™ RecoverAll™ Multi-Sample RNA/DNA Isolation Workflow (Thermo Fisher Scientific, Cat. No. A26135). Extracted RNA and DNA were quantified using the Invitrogen™ Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Cat. No. Q33216). The Invitrogen™ SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific, Cat. No. 11754050) was used for reverse transcription.

Libraries were prepared from 10 ng DNA and 10 ng RNA with Oncomine Focus Assay (Thermo Fisher Scientific, Cat. No. A28548) reagents on the Ion OneTouch™ 2 and Ion OneTouch™ ES Systems. The Ion PGM™ Select Library Kit and Ion OneTouch™ Select Template Kit were used. Six samples were run on the Ion 318™ Select Chip using the workflow described in the user guide, with each sample containing 80% DNA and 20% RNA, for a total of 12 barcodes. Sequencing was performed using the Ion PGM™ Select Sequencing Kit and Ion 318™ Select Chip Kit on the Ion PGM System.

### Data analysis

Data analysis was performed using Torrent Suite™ Server (ver. 5.0) and Ion Reporter™ Server (ver. 5.0), which includes the Oncomine™ Variant Annotation Tool (ver. 2.2). All variant types were included to assess correlation for precision and reproducibility.

Performance was computed using the following formulas:

Positive predictive value (PPV) = (CD)/(CD + FD)

Negative predictive value (NPV) = (CN)/(CN + FN)

Accuracy = (CD + CN)/(CD + CN + FN + FD)

Sensitivity = (CD)/(CD + FN)

Specificity = (CN)/(CN + FD)

(CD = confirmed detection, CN = confirmed nondetection, FD = false detection, FN = false non-detection)

## Results

### DNA sequencing performance

Five replicates of the Acrometrix Oncology Hotspot Control were sequenced on a single Ion 318 chip to assess detection of 69 annotated variants. A variant was called as detected if it was within the expected allele frequency range (either 5–15% or 15–35%) based on the manufacturer's characterization. Table 2 shows the concordance for each replicate run compared to the 69 variants used in the analysis.

**Table 2. Concordance of variants in the Acrometrix Oncology Hotspot Control with the expected allele frequency range.**

| Run | Number detected within allele frequency range | Number not detected within allele frequency range | Concordance |
|---|---|---|---|
| 1 | 69 | 0 | 100% |
| 2 | 68 | 1 | 99% |
| 3 | 68 | 1 | 99% |
| 4 | 69 | 0 | 100% |
| 5 | 68 | 1 | 99% |

The average concordance was 99%. The 3 variants not in range were all detected and only slightly below the 5% minimum allele frequency reported by the manufacturer.

For the 8 mutations assessed in the Quantitative MultiPlex Reference Standard, the expected vs. observed allele frequency is shown in Figure 2. The correlation coefficient was 0.99.

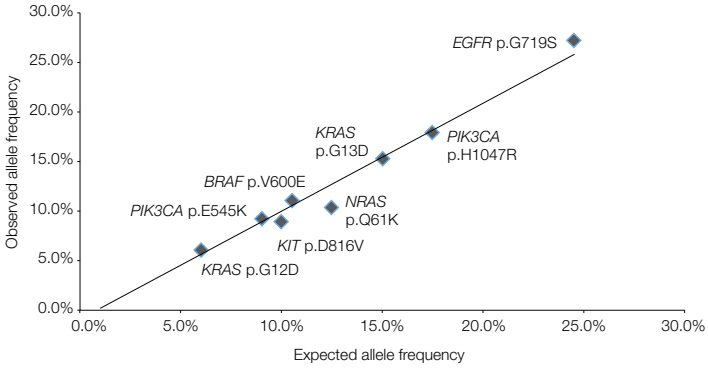


**Figure 2. Expected vs. observed allele frequency in the Quantitative Multiplex Reference Standard.**

The metrics for depth and uniformity of coverage were established across all target areas using 56 FFPE samples. The thresholds and observed ranges are shown in Table 3. Minimum and maximum metrics are based on confirmed detected variants in FFPE samples. The thresholds are based on analytical sensitivity and LOD analysis. The minimum number of variant reads for SNVs and indels was 250.

**Table 3. DNA sequencing metrics observed across 56 FFPE samples.**

| Metric | Threshold | Minimum observed* | Maximum observed* |
|---|---|---|---|
| Tumor reads | NA | 3M | 4.8M |
| Tumor percent | 0% | 10% | 100% |
| Mean depth | 850 | 849 | 4,234 |
| Uniformity of coverage | 75% | 74% | 100% |
| Mutation reads | 250 | 143 | 8,141 |
| Mutation allele frequency | 5% | 6% | 100% |
| Raw copy number | 4.5 | 3.9 | 46.4 |

* Variants observed below threshold are not included in the performance analysis.

To maximize representation of the complete Oncomine Focus Assay workflow from extraction to sequencing, FFPE samples were used to evaluate accuracy, sensitivity, and specificity. Fifty-six FFPE samples (Table 1, methods section) were used in this assessment. The detected variants for each sample were confirmed by either pre-characterization (i.e., known cell line or characterization by commercial vendor) or testing using an orthogonal method. Confirmation of variants was conducted by the following: SNVs and indels by Sanger sequencing and CNVs by fluorescence *in situ* hybridization (FISH). The results are shown in Table 4.

**Table 4. DNA sequencing performance analysis.**

| Number of variants | SNV | Indel | CNV |
|---|---|---|---|
| Confirmed, detection | 36 | 4 | 11 |
| Confirmed, nondetection | 60 | 12 | 8 |
| Confirmed, false detection | 1 | 0 | 0 |
| Confirmed, false nondetection | 0 | 0 | 0 |
| PPV | 97% | 100% | 100% |
| NPV | 100% | 100% | 100% |
| Accuracy | 99% | 100% | 100% |
| Sensitivity | 100% | 100% | 100% |
| Specificity | 98% | 100% | 100% |

The number of variants detected using NGS with the Oncomine Focus Assay exceeded those for which there is an orthogonal method available for testing. Additional variants detected by the Oncomine Focus Assay could not undergo confirmatory testing, as orthogonal tests are not available for some indels and CNVs. No variants detected by orthogonal methods were missed by NGS, hence no testing was required in the confirmed, false nondetection category.

## RNA sequencing performance

The thresholds in Table 5 were established based on internal testing and analysis. Threshold metrics are derived from the LOD studies using the H2228 cell line (*EML4–ALK* fusion).

**Table 5. RNA sequencing metrics.**

| Metric | Threshold | Minimum | Maximum |
|---|---|---|---|
| Tumor percent | 0% | 10% | 100% |
| Total mapped fusion reads | 10,000 | 131,197 | 198,759 |
| Fusion reads | 20 | 3,857 | 130,792 |

All detected variants (Table 6) exceeded the established thresholds in Table 5. Confirmation of variants was conducted by qPCR analysis and FISH in limited cases.

**Table 6. RNA sequencing performance analysis.**

| Number of variants | Fusion |
|---|---|
| Confirmed, detection | 6 |
| Confirmed, nondetection | 20 |
| Confirmed, false detection | 0 |
| Confirmed, false nondetection | 1 |
| PPV | 100% |
| NPV | 95% |
| Accuracy | 98% |
| Sensitivity | 86% |
| Specificity | 100% |

The fusions or variants that were detected as well as confirmed were *SCL45A3–ETV1*, *TMPRSS2–ERG*, *PTPRZ1–MET*, *SDC–ROS1*, *EGFRvIII*, and *TPM3–NTRK1*. The one false-negative variant (confirmed, false non-detection) was a *GOPC–ROS1* fusion that had been previously detected by NGS and confirmed using qPCR. A possible explanation for not observing this fusion could be tumor heterogeneity, though further testing is needed to determine the root cause.

## Limit of detection, CNV

The cell lines H1993 (*MET* and *CDK6* amplification) and H647 (*MYC* amplification) were diluted in order to adjust the copy number or tumor content. The diluted samples were tested with the Oncomine Focus Assay to determine the detection status at various copy number levels. The copy number gains assessed were 4.5 to 25. In order to determine the raw copy number instead of a tumor copy number, the cellularity was set to 100% for each of the dilution levels.

The analytical sensitivity was 100% at all dilutions with a raw copy gain of 4.5 or greater. The percent of tumor content impacts LOD for tumor copy number. A pathology assessment of the test sample for percent tumor is recommended. Figure 3 shows the relationship between the two and provides guidance for assessing the LOD against percentage of tumor content.

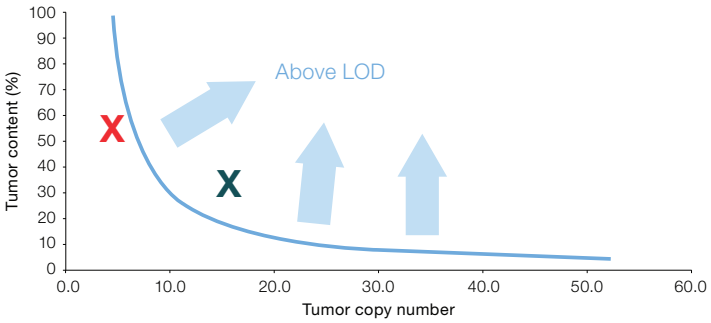


**Figure 3. Relationship between tumor content, tumor copy number, and LOD.**

## Limit of detection, SNVs, and indels

The cell lines H647, HCT116, and SKMEL-1 with known SNVs as well as cell line HCC4006 with known indels were diluted to various target allele frequencies (15%, 10%, 7.5%, 5%). The dilution levels were tested with the Oncomine Focus Assay to determine detection status. Two operators performed the study, with operator 1 performing two replicates for each level. The analytical sensitivity was found to be 100% at an LOD of 5% or greater allele frequency.

### Limit of detection, fusions

Cell line H2228, with an *EML4–ALK* fusion, was diluted to vary the amount of reads per targeted fusion. The fusion copy number at each dilution level was determined using 8 replicate runs of a single-analyte qPCR assay. The dilution levels were tested with the Oncomine Focus Assay to determine the detection status at various read levels. The analytical sensitivity and precision were 100% at an LOD of 225 fusion copies (Table 7).

**Table 7. Analytical sensitivity of gene fusion detection.**

| Dilution | Fusion copies (qPCR) | Sensitivity |
|---|---|---|
| 1 | 1,474 | 100% |
| 2 | 255 | 100% |
| 3 | 144 | 88% |
| 4 | 96 | 88% |
| 5 | 47 | 88% |

### Precision and reproducibility

Cell lines with known variants (SNVs, indels, and CNVs; see methods section) were diluted to various target allele frequencies (15%, 10%, 7.5%, 5%), and were tested with the Oncomine Focus Assay to determine detection status at the different levels. Two operators performed the assay and each operator ran all samples in duplicate.

The correlation coefficient was calculated for the following combinations for all variant types using relevant output (e.g., allele frequency, copy number):

- **Precision (repeatability)**: operator 1 – replicate 1 vs. operator 1 – replicate 2
- **Reproducibility**: operator 1 – replicate 1 and operator 1 – replicate 2 vs. operator 2 – replicate 1

As shown in Table 8, the Oncomine Focus Assay is highly reproducible and precise.

**Table 8. Precision and reproducibility of variant detection.**

| Variant category | Cell line | Variant | Precision (repeatability) | Reproducibility |
|---|---|---|---|---|
| SNV | H647 | *KRAS* p.G13D | 99% | 99% |
| SNV | HCT116 | *PIK3CA* p.H1047R | 98% | 99% |
| SNV | HCT116 | *KRAS* p.G13D | 97% | 99% |
| SNV | SKMEL-1 | *BRAF* p.V600E | 98% | 99% |
| Indel | HCC4006 | *EGFR* exon 19 deletion | 98% | 99% |
| CNV | H1993 | *CDK6* amplification | 98% | 98% |
| CNV | H1993 | *MET* amplification | 99% | 99% |
| CNV | H647 | *MYC* amplification | 99% | 99% |

## Summary and conclusion

A summary of results is shown in Table 9. The results indicate that the Oncomine Focus Assay is a precise, reproducible, sensitive, and accurate NGS assay for the detection of somatic genetic variants.


### Reference

1. Hovelson DH, McDaniel AS, Cani AK et al. (2015) Development and validation of a scalable next-generation sequencing system for assessing relevant somatic variants in solid tumors. *Neoplasia* 17:385–399.


**Table 9. Summary of sequencing results using the Oncomine Focus Assay.**

| Category | Result |
|---|---|
| Performance characteristics, Acrometrix Oncology Hotspot Control (69 variants): concordance, 5 runs | 99% |
| Performance characteristics, Acrometrix Oncology Hotspot Control: variant allele frequency correlation coefficient between 5 runs | 98% |
| Performance characteristics, Quantitative Multiplex Reference Standard (8 mutations): expected vs. observed allele frequency, 1 run | 99% |
| Analytical sensitivity (LOD): SNV | 100% at ≥5% allele frequency |
| Analytical sensitivity (LOD): indel | 100% at ≥5% allele frequency |
| Analytical sensitivity (LOD): CNV | 100% at ≥4.5 raw copy number |
| Analytical sensitivity (LOD): RNA fusion | 100% at ≥255 fusion copies |
| Precision (within run) and reproducibility (between runs): SNV | 98%; 99% |
| Precision (within run) and reproducibility (between runs): indel | 98%; 99% |
| Precision (within run) and reproducibility (between runs): CNV | 99%; 98% |
| Precision (within run): RNA fusion | 100% at ≥255 fusion copies |
| Accuracy and specificity: SNV (above LOD) | 99%; 98% |
| Accuracy and specificity: indel (above LOD) | 100%; 100% |
| Accuracy and specificity: CNV (above LOD) | 100%; 100% |
| Accuracy and specificity: RNA fusion (above LOD) | 98%; 100% at ≥255 fusion copies |

ion torrent

Find out more at **thermofisher.com/oncomine**

ThermoFisher SCIENTIFIC